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Application of Electronmicroscopy, Enzyme Immunoassay, and RT-PCR to Monitor an Outbreak of Astrovirus Type 1 in a Paediatric Bone Marrow Transplant Unit

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During 1997, an extensive outbreak of astrovirus occurred in a unit where paediatric patients were being treated for leukaemias and inherited immune deficiency disorders. Prolonged shedding of virus for many months following infection was demonstrated in three patients who had undergone bone marrow transplantation. Comparison of reverse transcription-polymerase chain reaction (RT-PCR), enzyme immunoassay (EIA), and electronmicroscopy (EM) to monitor the outbreak showed that many subclinical infections, mainly in children aged > 3 years could only be detected by RT-PCR. Use of RT-PCR revealed that several patients were infected earlier and shed virus for longer than by using EM or EIA. The virus responsible for the outbreak was identified as HAstV-1 and was shown to have a sequence that differed from a strain obtained in 1988. ***J. Med. Virol. 57:313–321, 1999.***

KEY WORDS: SCID; diagnosis; bone marrow transplantation

INTRODUCTION

Astroviruses were first described by Appleton and Higgins [1975], as the result of an investigation into the cause of an outbreak of diarrhoea in a mother and baby unit in the south of England. Since then, eight antigenic types, HAstV 1–8, have been identified in humans and cases recorded throughout the world [Cubitt, 1996]. Outbreaks and sporadic cases have been reported in all age groups [Ashley et al., 1978; Gray et al., 1987; Herrmann et al., 1991; Midthun et al., 1993; Oishi et al., 1994; Belliot et al. 1997], but infection is believed to occur most frequently in young children. Symptoms of infection are typically vomiting and diarrhoea, which are generally mild and of short duration. However, in immune compromised and immune deficient patients, prolonged shedding of astrovirus has been demonstrated in association with symptoms of diarrhoea [Grohmann et al., 1993; Cox et al., 1994; Noel and Cubitt, 1994].

Cox et al. [1994] conducted a prospective study into the aetiology and outcome of diarrhoea after bone marrow transplantation (BMT). There were 150 episodes of acute diarrhoea recorded in 126 of 296 patients included in the study. The majority of diarrhoea episodes, 72 (48%), were associated with graft-versus-host disease (GVHD). Only 20 (13%) episodes were attributed to gastrointestinal infection, most (35%) of which were associated with astrovirus infection.

Reverse transcription-polymerase chain reaction (RT-PCR) was applied by Mitchell et al. [1995] to monitor an outbreak of diarrhoea in a child care center that demonstrated that RT-PCR was more sensitive than an enzyme immunoassay (EIA) for detection of astrovirus. Evidence of infection was demonstrated earlier and shedding of virus shown to persist when RT-PCR was applied to examine sequential samples and many asymptomatic cases were identified.

The present study describes an outbreak of HAstV-1 in a paediatric BMT unit and compares the sensitivity of three diagnostic tests (electronmicroscopy [EM], EIA, and RT-PCR) for detecting infection. Problems associated with monitoring and controlling the outbreak are discussed.

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CLINICAL SETTING

The outbreak occurred in a unit where paediatric patients were being treated for haematologic malignancies and inherited immune deficiency disorders, five of whom had undergone BMT (Table I). The unit is comprised of 19 isolation cubicles, each containing its own separate toilet and washing facilities. Patients undergoing BMT are cohorted in positive pressure cubicles (#1–3 and #6–10), which are accessed through airlocks and vented to the outside environment.

The Outbreak

A case of astrovirus infection on the unit was detected by EM on 11 December 1996 in an 11-month-old female with symptoms of diarrhoea. She was hospitalized on the ward for the previous 5 months, during which time several stool samples from her had been examined by EM and found to be negative. Screening of all patients in the unit by EM revealed no further cases of astrovirus infection until 3 January 1997 when a child, Case 1, (Table I, Figs. 1, 2A) with chronic myeloid leukaemia (CML) presented with diarrhoea (8×/day) and vomiting and had persistently loose stools for the following 6 weeks. Examination of faecal samples by EM showed that he continued to periodically shed large numbers of astrovirus particles for a further month following cessation of symptoms (Fig. 2A). During the next 3 weeks, a further seven patients in the unit were shown by EM and/or EIA to be shedding astrovirus particles, all of them had diarrhoea/loose stools and or vomiting. Another case (Case 5) was recorded in a patient who had been in the unit from 9th to 15th January 1997 and was admitted to another hospital on the following day with vomiting and diarrhoea. A faecal sample obtained at the time of admission was examined by EM at another hospital and found to contain astrovirus (Table I, Fig. 1).

All five astrovirus-infected BMT patients (Table I, Figs. 1, 2A, B, D) presented with symptoms of vomiting and /or diarrhoea. Case 1 had diarrhoea and vomiting for 2 weeks but continued to have loose mucoid stools for a further month that was associated with GVHD. Onset of astrovirus-associated symptoms occurred within 3 weeks of BMT in four patients; the fifth case occurred 3 months after transplantation.

Cases 6, 9, and 10 had diarrhoea, two to four times/day lasting for 1–6 days, but astrovirus was not detected by EM or EIA. HAstV-1 infections were identified retrospectively when samples were tested by RT-PCR.

The last patient to present with symptoms was on 27 January 1997. At this time, the ward had been closed to new admissions. The staff was reminded of the need to adhere strictly to the guidelines for handwashing and teaching sessions were provided for the cleaning staff. On 12 February 1997, the entire unit was cleaned thoroughly with hot soapy water and surfaces cleaned with alcohol wipes. The following day the unit was inspected by members of the control of infection team. However, five patients (Case 1, 3, 7, 8, and 11) who remained in the unit continued to shed virus for prolonged periods, 31–130 days (Figs. 1 and 2A, C, D).

Screening of faecal samples from all 11 patients in the unit on 19 February 1997 by EM and EIA failed to reveal any new cases and the ward was reopened to admissions.

MATERIALS AND METHODS

Bacteriological and Virological Examination

Faecal samples from patients with symptoms of diarrhoea were cultured routinely for the presence of bacterial pathogens (Salmonella, Shigella, *Campylobacter* sp. *Clostridium difficile*) and examined by EM for the presence of virus particles. *Clostridium difficile* isolates were screened for toxin production by cell culture. Once an outbreak of astrovirus infection on the unit became apparent, stool samples were obtained from all symptomatic and asymptomatic patients. Samples were negatively stained with potassium phosphotungstic acid, pH 6.4, and examined by direct EM and in a commercial astrovirus EIA (IDEIA Astro, Dako Ltd, Ely, Cambs, UK) according to the manufacturer's instructions. A positive EIA result was recorded when samples gave A450-nm readings of > 0.15 + kit control negative. Stools from patients found to be excreting virus were collected as often as possible until three consecutive samples were found to be negative by both EM and EIA, at which point the patients were considered to have ceased virus shedding. The policy of screening stool samples by EM from all patients on the unit on a weekly basis was continued.

Screening of Hyperimmune Gammaglobulin Prior to Oral Administration

Hyperimmune gammaglobulin (Sandos, Switzerland) was used to screen for the presence of astrovirus-specific IgG in an EIA using baculovirus-expressed HAstV1 capsid protein [Kriston et al., 1996].

Retrospective Screening of all Faecal Samples From the Unit by RT-PCR

A subset of EM and EIA astrovirus-positive or -negative faecal samples from this outbreak and from unrelated sporadic diarrhoeal episodes were blind tested using an astrovirus group-specific primer pair and HAstV-1- and HAstV-2-specific primers. The initial results demonstrated a 559-bp product using an HAstV-1-specific primer pair of AV3/Mon2, indicating that HastV-1 was present in the BMT unit. Samples (N = 176) were randomized and coded prior to testing with this primer pair for the remainder of the samples obtained from the unit. The following primers and reaction conditions were used: Oligonucleotide primers for RT-PCR were chosen from the 3′ terminus of the astrovirus genome of all eight types. Positive sense primers PR6257 = 5′-ACA TTG CCC AGA ATT TC; AV3 5′-ATG CCT TTG CCT GAG TCC AC [Jonassen et al., 1993] and Mon 67 [Mitchell et al., 1995] were used. The three second-strand primers produced the follow-

TABLE I. Characteristics of 20 Patients Involved in an Astrovirus Outbreak on a Paediatric BMT Unit and Comparison of EM, EIA, and RT-PCR Results for Detecting Infection Onset

Case	Age	Cubicle	Diagnosis	Date of BMT	Symptoms & Duration	Admitted	1st pos	EM	EIA	RT-PCR	Serotype
	11m				*D*		*11.12.96*	***Astro***	***1.8 +***	*Neg*	*Type 4*
1	12m	**10**	CML	07/01/97	D, 42 days	27/12/96	03.01.97	**Astro**	**0.7 +**	**Pos****	**Type 1**
2	29m	**8**	Hurler's	7/96; 18/12/96	D, 7 days	10/12/96	06.01.97	**Astro**	**1.7 +**	**Pos****	**Type 1**
3	23m	4	Neuroblastoma		D, 45 days	26/12/96	13.01.97	Neg	0.03 –	**Pos***	**Type 1**
4	21m	**7**	AML	20/12/96	D & V, 6 days	09/12/96	15.01.97	Neg	0.04 –	**Pos***	**Type 1**
5	20m	19	ALL		D, V	03/10/96	16.01.97	**Astro**	NA	NA	**NA**
6	**8y**	13	ALL		D, 1 day	03/01/97	23.01.97	Neg	0.02 –	**Pos***	**Type 1**
7	9m	**1**	SCID	08/01/97	D, 128 days	02/01/97	23.01.97	**Astro**	**2.2 +**	**Pos****	**Type 1**
8	3m	**14**	AML		D, 98 days	03/01/97	23.01.97	Neg	0.04 –	**Pos***	**Type 1**
9	19m	18	AML		D, 6 days	27/12/97	24.01.97	Neg	0.14 –	**Pos****	**Type 1**
10	6y	6	AA		D, 5 days	18/01/97	25.01.97	Neg	0.13 –	**Pos****	**Type 1**
11	16m	**10**[a]	ALL	11/96	D, 12 days	18/01/97	27.01.97	Neg	0.04 –	**Pos***	**Type 1**
12	12m	**15**	AML		D, 14 days	16/12/97	27.01.97	**Astro**	**2.1 +**	**Pos****	**Type 1**
	7y	2	HG		D, V		28.01.97	SRSV	0.03 –	Neg	Calici GpII
Ward closed to new admissions. Cleaned with hot soapy water and alcohol wipes; inspected and reopened 13/02/97.											
13	30m	**1**[a]	Neuroblastoma		Asymptomatic	13/02/97	18.02.97	Neg	0.19 +/–	**Pos*****	Type 1
14	**6y**	**7**[a]	ALL		Asymptomatic	23/01/97	18.02.97	Neg	0.14 –	**Pos****	Type 1
15	**3y**	16	AML		Asymptomatic	13/02/97	20.02.97	Neg	0.04 –	**Pos***	Type 1
16	**9y**	**8**[a]	CID		Asymptomatic	27/01/97	23.02.97	Neg	0.02 –	**Pos***	Type 1
17	**11y**	**14**[a]	AML		Asymptomatic	14/01/97	23.02.97	Neg	0.04 –	**Pos***	Type 1
18	10m	NA	HPL		Asymptomatic	20/03/97	27.03.97	Neg	0.17 +/–	**Pos****	Type 1
19	18m	**15**[a]	AML		Asymptomatic	19/03/97	29.03.97	Neg	0.19 +/–	**Pos*****	Type 1
20	**7y**	9	ALL		Asymptomatic	01/03/97	03.04.97	Neg	0.12 –	**Pos***	Type 1

BMT, bone marrow transplantation; EM, electronmicroscopy; EIA, enzyme immunoassay; RT-PCR, reverse transcription-polymerase chain reaction; CML, chronic myeloid leukaemia; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; SCID, severe combined immune deficiency; AA, aplastic anaemia; HG, hypogammaglobilinaemia; CID, combined immune deficiency; HL, Hodgkin's Lymphoma; D, diarrhoea stools; V, vomiting.
Asterisks refer to strength of band (*, weak band; ****, strong band).
Positive results for astroviruses are shown in bold.
An unrelated case of astrovirus type 4 on the unit is shown in italics.
[a]Second patient infected in cubicle.

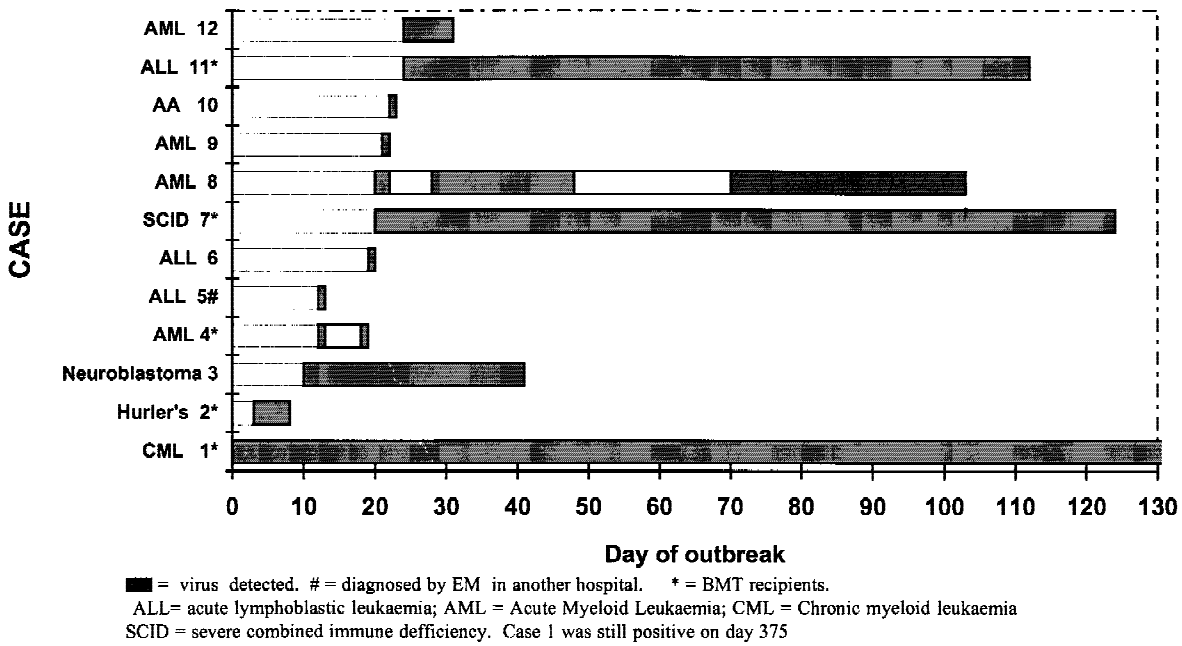

Fig. 1. Time course of astrovirus excretion in patients infected during the first wave of the outbreak; dates of onset 3–27 January 1997.

ing RT-PCR products: PR6257, a 540-bp product only for HAStV-2; primer AV3, a 559-bp product only from HAstV-1; and Mon 67, an 89-bp product from all astrovirus types. Positive (HAstV-1 and HAstV-2), negative (uninfected cell culture extract), and water controls were included in each run.

Viral RNA was extracted from faecal samples which had been stored at 4°C using TRIZOL Reagent (Gibco BRL, Bethesda, MD) following suspension of stool in nuclease-free water and extraction with an equal volume of Genetron (Sigma Ltd, Dorset, UK). Reverse transcription occurred in a 50-μl volume, using 5 μl of extracted RNA in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1.25 mM of each of four dNTPs (Promega, Southampton, UK), 1 mM first-strand primer (Mon 2) [Mitchell et al., 1995] and 10 U of avian myeblastosis reverse transcriptase (Promega). The reaction was incubated at 60 min at 42°C, after which PCR occurred in 100 μl with the same buffer containing 0.5 mM dithiothreitol, 0.625 mM of each of four dNTPs, 5% dimethyl sulfoxide, 0.5 μM first strand primer, and 2.5 U of Taq polymerase (Promega). Thirty cycles of the following conditions were repeated: 1 min at 90°C, 2 min at 40°C, and 1 min at 72°C. Reaction products were analysed by electrophoresis in 1% agarose gels prepared in Tris-borate ethylenediamine tetraacetic acid (TBE) buffer and visualized by staining with ethidium bromide and illumination with ultraviolet (UV) light.

PCR products obtained from two patients (Cases 1 and 8) were cloned and sequenced and compared with the sequence obtained from a HAstV-1 strain (A2/88/UK) obtained from a child in 1988 [Willcocks et al., 1994].

RESULTS

Astrovirus particles with typical surface morphology were detected by EM in stools from one patient on 11 December 1996 that was identified as HAstV-4 by immune EM. After a period of 3 weeks when no astrovirus cases were recorded in the unit, nine patients who had loose stools were shown by EM and/or EIA to have become infected with astrovirus (3–27 January 1997), (Fig. 1). Subsequent examination of sequential samples by RT-PCR and sequencing of the amplified products showed that the virus was a strain of HAstV-1. Several children (Cases 2, 3, 4, 8, and 11) were shown by RT-PCR to shed virus for longer (6, 3, 7, 59, and 13 days, respectively) than was demonstrated by either EM or EIA (Table I , Figs. 1, 2B, C, D). Cases 3, 8, and 11 were found to be infected earlier (9, 9, and 13 days, respectively) than was predicted by either EM or EIA. Application of RT-PCR revealed three additional cases (#6, 9, and 10) of astrovirus infection in the first wave of the outbreak that were not detected by EM or EIA. Screening of faecal samples from all 11 patients on the ward by EM and EIA following closure to admissions and cleaning of the unit revealed no new cases. This finding indicated that the control measures had been successful. However, retrospective examination of faecal samples collected during February, March, and April 1997 by RT-PCR detected a further eight cases. Faecal samples from three patients that gave strongly positive (**/***) results by RT-PCR had previously given equivocal results in the EIA (Table I). A significant number (5 of 7, $P = .04$) of the patients infected during the second wave of the outbreak were located in cubicles that had been occupied previously by children excreting astrovirus in the first wave of infection.

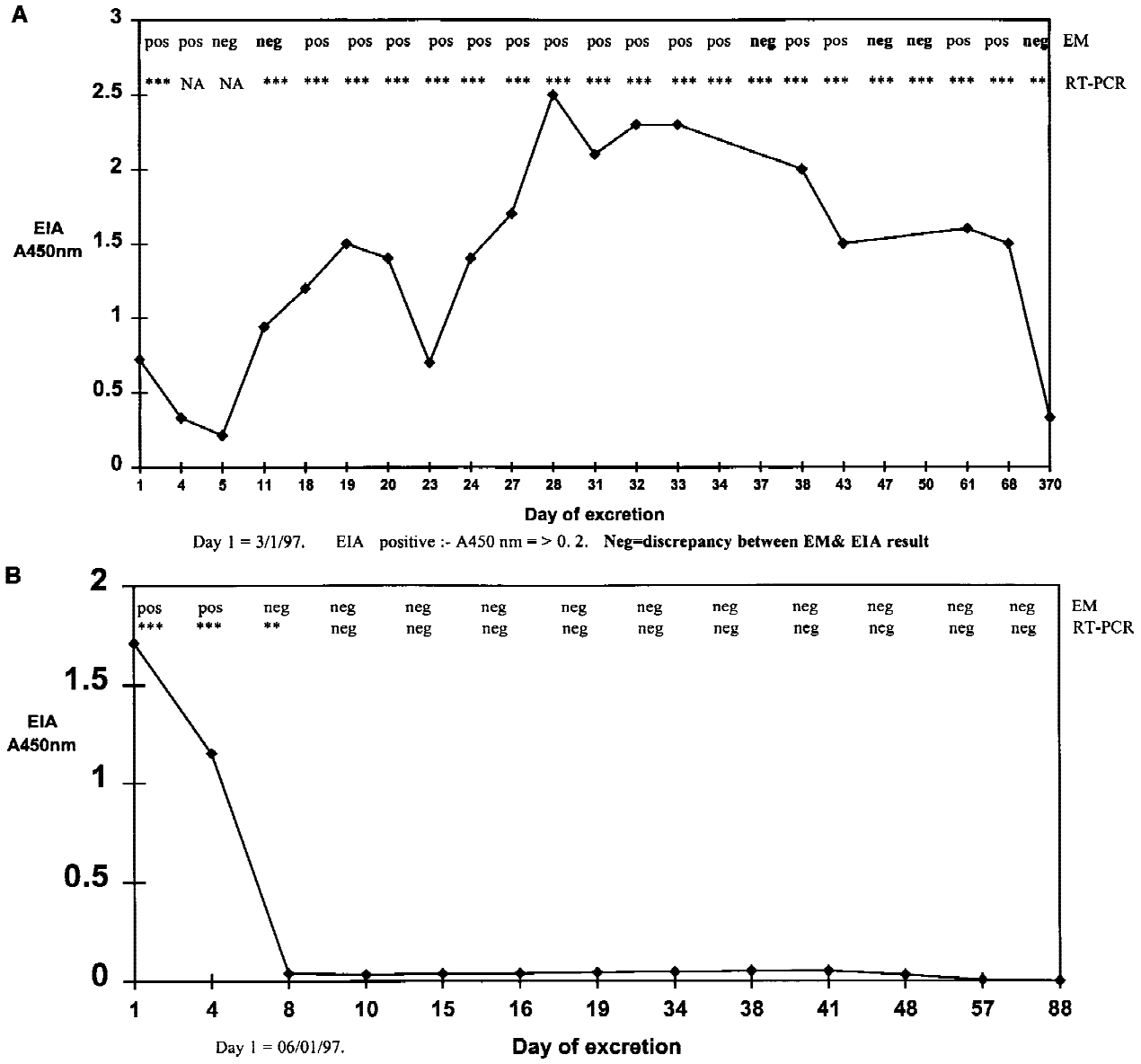

Fig. 2. Comparison of electronmicroscopy, enzyme immunoassay, and reverse transcription-polymerase chain reaction (RT-PCR) to monitor astrovirus excretion. **A:** Case 1. Patient aged 12 months with chronic myeloid leukaemia (CML) given a bone marrow transplant (BMT) on day 5, showing prolonged excretion for over a year. **B:** Case 2. Patient aged 29 months with Hurler's syndrome given a BMT on 18/12/96. (*Figures continues.*)

Subsequent testing of faecal samples by RT-PCR from all the patients in the unit on two occasions during April and May 1997 showed no evidence of further cases, indicating that the virus had not become endemic in the unit. Weekly screening of patients in the unit by EM failed to reveal any further cases of astrovirus infection until February 1998, when a patient with acute myeloid leukaemia (AML) was found to be shedding large numbers of virus particles. The faecal sample produced a strong positive signal in the EIA. This virus failed to produce an RT-PCR product with HAstV-1-specific primers AV3/Mon2, indicating either a different type or a different strain of HAstV.

Comparison of the EM, EIA, and RT-PCR results appeared to correlate with the amount of PCR product (Table I, Fig. 2). Samples detected by EM that had A450-nm values of greater than 0.3 in the EIA uniformly produced strong reactions (***) by RT-PCR. Samples that were EM negative and EIA negative but had A450-nm values (0.15–0.20) considerably above the background levels (0.02–0.08) but below the cut-off of the assay produced clearly evident (**) RT-PCR products. Some samples that produced A450-nm values indistinguishable from the background in the EIA produced weak RT-PCR (*) reactions (Table I, Fig. 2). Previous and subsequent samples collected from asymptomatic patients were found to be negative by all three tests (e.g., Case 14, Fig. 2E).

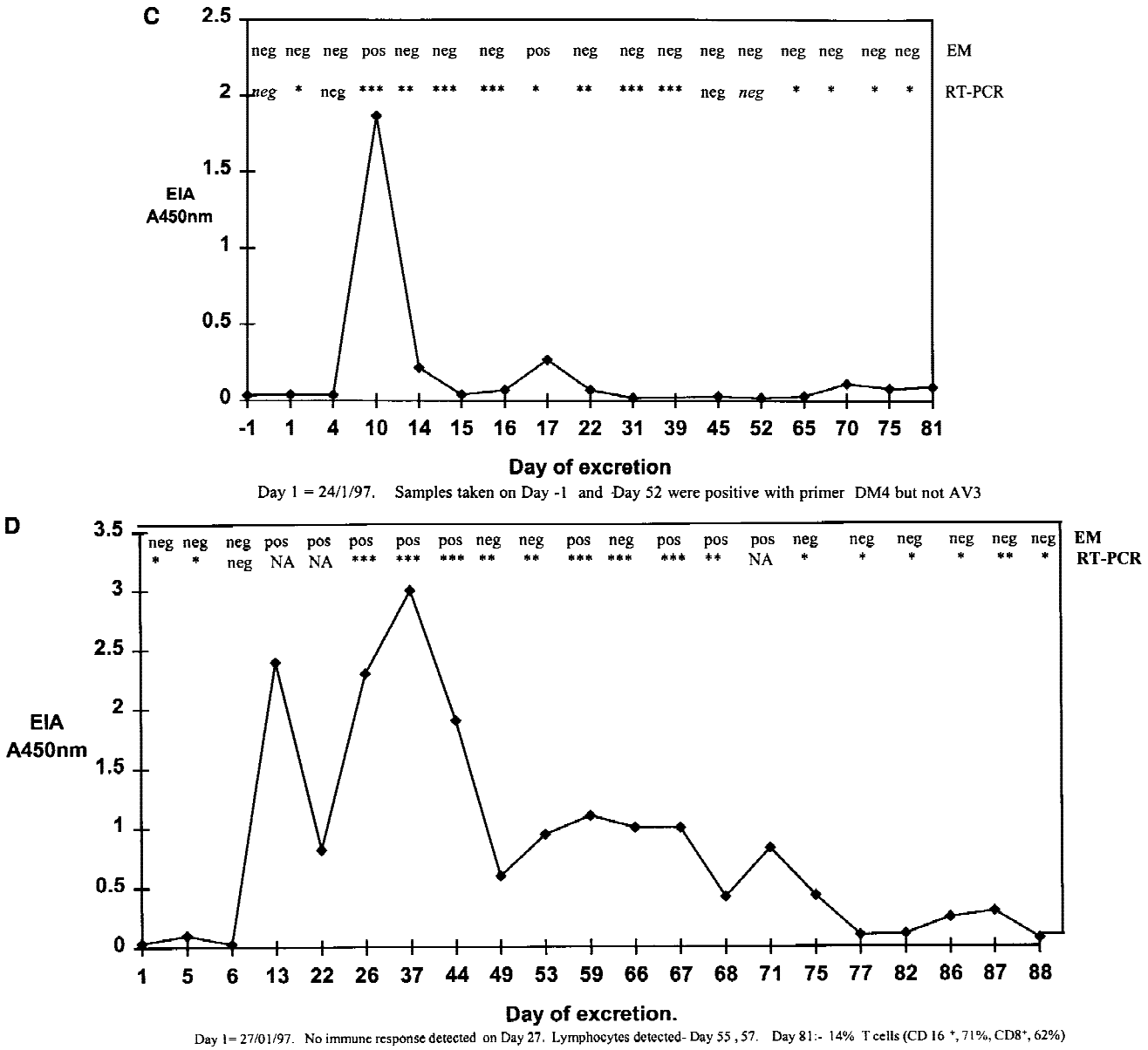

Fig. 2. *Continued.* **C:** Case 8. Patient aged 3 months with acute myeloid leukaemia (AML). **D:** Case 11. Patient aged 16 months with all, 2 months post-BMT, showing decline in virus levels once a T-cell response could be demonstrated. (*Figures continues.*)

The precise duration of astrovirus shedding in five patients with diarrhoea/loose stools but who had not received a BMT could not be ascertained, as specimens were not obtained from all patients on a daily basis. However, the duration was generally short (1–7 days), with the exception of Case 8 (Fig. 2D), who periodically shed virus for more than 81 days. In contrast, three of five patients (Cases 1, 7, and 11) who had undergone BMT with prolonged and more severe symptoms continued to shed variable amounts of virus for up to 1 year (Figs. 1, 2A, C, D). The other two BMT patients excreted virus for 8–10 days. All the asymptomatic patients (Cases 13–20) were found to be positive by RT-PCR only and on a single occasion, except a child aged 18 months (Case 20) who was shown by RT-PCR to shed virus for 9 days.

Oral administration of hyperimmune gammaglobulin with a high titer (1:128,000) of IgG to HAstV1 was shown by EM to have caused virus particles to aggregate into large immune complexes in faecal samples, but failed to clear the infection. This result was particularly apparent in Case 1 and probably accounts for the failure to detect virus by EM on several occasions when both the EIA and RT-PCR produced strongly positive reactions (Fig. 2A).

In one long-term excretor (Case 11), production of T cells (CD 16 $^{+}$ and CD8^{+}) was associated temporally with decreased viral levels, which could only be detected by RT-PCR (Fig. 2D).

A further three symptomatic patients on the unit were shown by EM to be excreting enteric viruses in their stools (1 small, round, structured virus; 1 adeno-

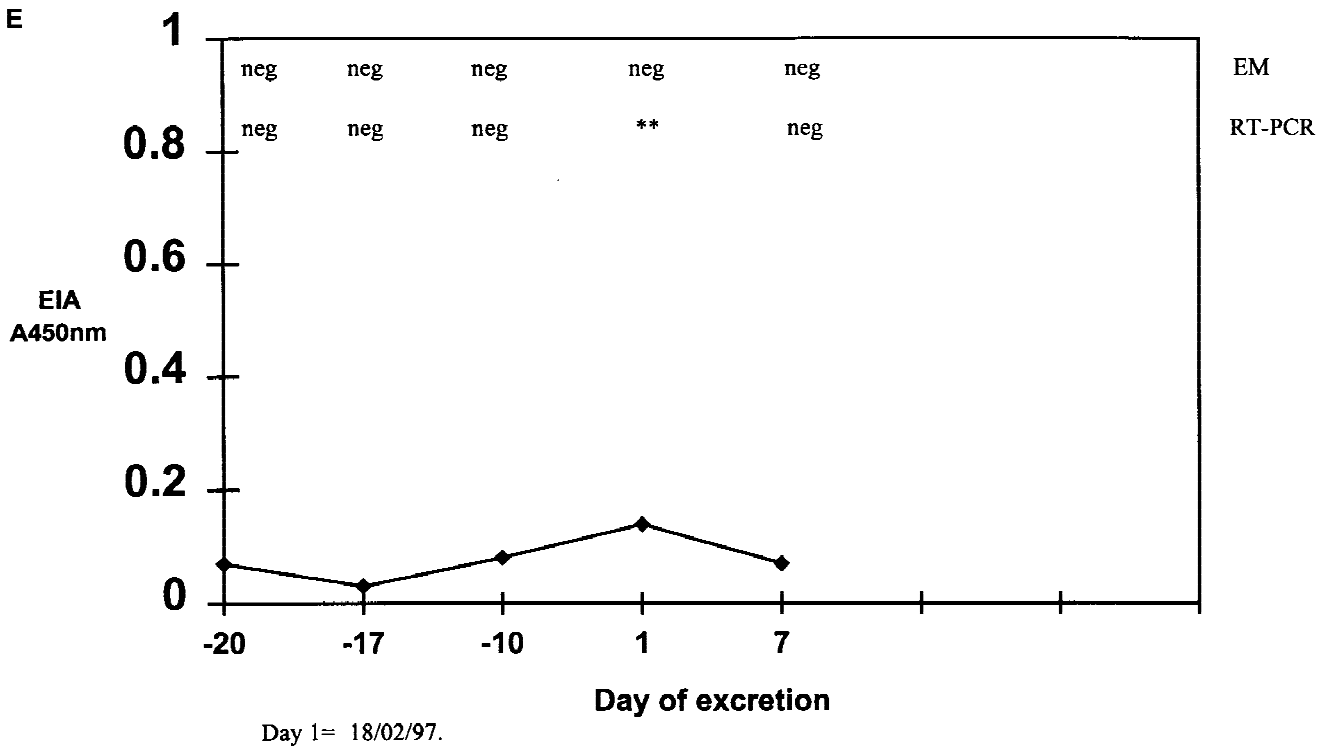

Fig. 2. *Continued.* **E:** Case 14. An asymptomatic patient aged 6 years with relapsed acute lymphoblastic leukaemia (ALL), showing transient excretion of low levels of astrovirus, only detected by RT-PCR.

virus, 1 rotavirus). Screening of samples from these patients by EIA and RT-PCR failed to show any evidence of concurrent infection with astrovirus. However, a patient who was infected with adenovirus in January and then discharged home, subsequently became infected with astrovirus following readmission to the unit in February (Case 15, Table I).

Cloning of the RT-PCR products from samples obtained from Case 1 in February and from Case 8 in April and comparison of the sequences showed a single amino acid difference (0.6%). A single amino acid change was also detected in samples taken a week apart from Case 8. Alignment of the sequences with strain A2/88 (Genbank Accession #S68561) [Willcocks et al., 1994] showed 92.5% amino acid identity. An additional amino acid (asparagine) at position 690 was detected in samples from both patients. Therefore, the strains isolated in this outbreak were related but different from a HAstV-1 strain isolated 9 years earlier in the UK.

DISCUSSION

The results of the present study confirm the previous observations [Cox et al., 1994] that episodes of diarrhoea/ loose stools occur frequently in patients who have undergone BMT and that astroviruses and other enteric viruses, i.e., rotavirus, caliciviruses, and adenovirus, are a cause of intestinal infection. In both studies, astrovirus infections were acquired in a hospital, suggesting that asymptomatic carriage of the virus is common or that the virus persists in the environment for prolonged periods of time. Previous surveys have demonstrated that asymptomatic carriage of astrovirus occurs frequently [Scott et al., 1979; Kotloff et al., 1992; Mitchell et al., 1995] and virus particles are known to remain viable in the environment for prolonged periods of time [Noel and Cubitt, 1994; Abad et al., 1997]. It is of note that astroviruses are resistant to degradation at 56°C [Myint et al., personal communication], the temperature at which bedpan washes operate. Therefore, we speculate that this procedure could generate aerosols of astrovirus. Particular attention to handwashing and thorough cleaning of the unit with hot soapy water and alcohol wipes was associated temporally with the cessation of further cases of diarrhoea on the unit. Retrospective testing by RT- PCR, however, showed the presence of secondary astrovirus cases in cubicles that had been used previously to treat patients who had been excreting large amounts of virus. This finding suggests that astrovirus may have remained viable in the environment or that staff or visitors within the unit were transmitting the virus. An alternative explanation for the low levels of virus and short duration of virus shedding in the second wave of infection is that the majority of patients were older (>3 years) and therefore were likely to have been exposed previously to infection with HAstV-1 [Kriston et al., 1996; Koopmans et al., 1998]. Some support for this argument is shown by the apparently higher viral loads (i.e., RT-PCR **/***, EIA±) detected in the three

patients who were aged 17–30 months and the failure to detect virus by EM or EIA in two patients who were > 3 years old (Cases 6 and 10).

The application of EM and EIA to screen for the presence of astroviruses in stools demonstrated that both techniques had a similar level of sensitivity but EIA was found to be more practical for monitoring an extensive outbreak. RT-PCR testing demonstrated clearly that in some patients the concentration of virus being shed dropped below the level of detection (10^{5-7}/g) of EIA and EM on several occasions and subsequently rose above the threshold of detection. Therefore, to control an outbreak effectively, it is advisable that samples be examined on several occasions after a patient becomes asymptomatic and appears to have cleared the virus. The lack of transmission of rotavirus, calicivirus, or adenovirus infection in the unit despite the presence of infected patients during the period of investigation suggests that the outbreak may have been caused by the extremely high environmental load of astrovirus particles generated by immune deficient patients. A similar situation has been shown to exist in mouse colonies where explosive outbreaks of rotavirus (EDIM) occur once the viral loads in the environment become excessive [Kraft, 1957].

The difficulty of distinguishing which patients being treated on a BMT unit have diarrhoea/loose stools associated with therapy or GVHD rather than due to infection illustrates the necessity of surveillance to detect and control outbreaks. However, application of RT-PCR showed that the policy of screening faecal samples by EM or EIA was not sufficiently sensitive because many patients were proven to be infected several days prior to EM or EIA becoming positive and 10/20 cases (8 asymptomatic) would not have been detected at all. These numbers are similar to that in a closed population of children attending a day care centre during an astrovirus outbreak [Mitchell et al., 1995]. Therefore, there is a need to develop multiplex PCR techniques to detect a wide range of enteric pathogens in units treating immune compromised patients.

One report has described the successful intravenous use of gammaglobulin to ameliorate severe astrovirus-associated diarrhoea in a patient with Waldenstrom's macroglubinaemia [Bjorkholm et al., 1995]. However, in this study the use of orally administered hyperimmune gammaglobulin containing a high concentration of astrovirus-specific IgG antibodies aggregated virus particles into large immune complexes in faecal samples but failed to prevent viral replication. In addition, one patient (Case 11) who shed large amounts of astrovirus for more than 2 months decreased excretion following detection of T cells ($CD8^+$, $CD16^+$). The presence of large numbers of natural killer cells at the time suggests that they may be essential for eliminating infection, as has been suggested recently [Molberg et al., 1998]. Support for this theory comes from experiments on severe combined immune deficiency (SCID) mice infected with murine rotavirus in which infection was ablated by passive transfer of $CD8^+$ lymphocytes [Dharakul et al., 1990].

Further studies are needed to investigate the importance of CMI compared with humoral responses in the clearance and prevention of enteric infections, particularly now that vaccines are being introduced to combat viral diarrhoea (i.e., rotavirus).

Sequence analysis of 160 amino acids from the 3′ end of the protein (ORF2) demonstrated 92.5% identity with a strain obtained 9 years previously, indicating more variability within strains of HAstV-1 than has been reported previously [Noel et al., 1995]. The reason for this is that the RT-PCR detection and sequence analysis in this part of ORF2 spans the most hypervariable region rather than more well-conserved, nonstructural protein regions or the more conserved 5′ end of ORF2 [Willcocks et al., 1995]. Further studies are now in progress to determine the amount of variability between samples collected from the long-term excretors in the absence of immune pressure and to ascertain whether quasispecies exist.

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